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CYTOCHROME OXIDASE IN ANTERIOR AND POSTERIOR BLASTOMERES OF CIONA INTESTINALIS

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The cytochemical test for indophenol blue oxidase, the indophenol or "nadi" reaction, has long been used to demonstrate supposed gradients or localizations of this enzyme in a wide variety of embryonic material. A particularly striking localization of this enzyme, presumably cytochrome oxidase, has been claimed for the ascidian egg where the myoplasm, presumptive for the larval musculature, gives an intense reaction with the indophenol reagents (Ries, 1937, 1939; Reverberi and Pitotti, 1939).

Cytochemical tests for demonstrating localization of enzymes in living cells are open to serious criticisms. Differential rates of staining of cells or of areas of a cell could be due to a variety of factors other than differences in enzyme activity, and it is extremely difficult to evaluate the sources of error. The aim of the present investigation was to make quantitative measurements of cytochrome oxidase in the ascidian blastomeres which stained differentially with the indophenol reagents. At the four-cell stage, the ascidian embryo consists of anterior and posterior blastomeres, the latter giving a strong oxidase reaction. Techniques were developed for separating the two types of blastomeres in sufficient numbers for quantitative analyses of cytochrome oxidase by a microspectrophotometric method. The results reported below verify the conclusions reached by previous investigators using cytochemical methods.

Methods

Gametes were obtained from *Ciona intestinalis* by removing the test and opening the oviduct and vas deferens. Chorions of the unfertilized eggs were digested off with a protease solution (3% crude protease [1] in sea water) and the "naked" eggs were washed, transferred to agar-coated dishes and fertilized. An agar coat on all glassware is necessary to prevent sticking and subsequent cytolysis of the eggs. Several hundred first-cleavage blastomeres were separated from one another and in turn segregated by means of a breaking pipette from the remaining mass of eggs. Division of each isolated first-cleavage blastomere resulted in an anterior and posterior cell which were separated with the tip of a breaking pipette and transferred to separate dishes. Although they are identical in volume the an-

[1] Purchased from Worthington Biochemical Sales Co., Freehold, New Jersey.

terior and posterior blastomeres can be distinguished by the elongated shape and clear cytoplasmic "cap" of the posterior blastomere (Castle, 1896). These characteristics are temporary and identification and separation of the blastomeres must be performed rapidly.

Cytochrome oxidase was measured by a microspectrophotometric method originally described by Cooperstein and Lazarow (1950) and repetitiously by Hess and Pope (1953). Basically, the method consists of the spectrophotometric observation of the rate of oxidation of reduced cytochrome *c* with the Beckman spectrophotometer adapted for the use of microcuvettes (Lowry and Bessey, 1946).

In more detail, the method as used for determination of cytochrome oxidase in eggs is as follows. The eggs or blastomeres were transferred by means of a breaking pipette to a 0.1-ml. conical-shaped centrifuge tube. By light centrifugation the cells were forced to the end of the tube and excess sea water was then drawn off with a pipette. Four to five μl. of homogenization medium were added, and the eggs homogenized by drawing them in and out of a fine pipette having a bore less than the diameter of an egg. The homogenization medium consisted of 0.75–1.0% sodium desoxycholate in phosphate buffer. This has been shown to have a clarifying action on crude homogenates and apparently causes a dispersal of the cytochrome oxidase complex (Hess and Pope, 1953). An attempt was made to reduce deterioration of cytochrome oxidase after homogenization by including serum protein in the homogenization medium (Borei, 1950). Although with some types of material the added protein seemed to offer some protection, its inclusion was not essential in the present experiments. Loss of enzyme activity was reduced considerably by rapid homogenization of the eggs at 3 to 4° C. Temperature control was obtained by placing the homogenization tube in a previously chilled copper block with drilled holes for microscopic observation.

The above procedure required only 20 to 30 seconds and the homogenate was then added to 45 μl. of reduced cytochrome *c* solution (1.6×10^{-5} *M* cytochrome *c*, reduced with sodium hydrosulfite, in 0.04 *M* phosphate buffer, pH 7.2) contained in a 50-μl. microcuvette. The reactants were stirred with a fine glass rod and the change in optical density at 550 millimicrons was followed for 2.5 minutes in a Beckman spectrophotometer. At the end of this period a small amount of potassium ferricyanide was added and the optical density of completely oxidized cytochrome *c* was measured. The measurements were carried out at room temperature (22 to 24° C.).

With the concentration of reactants used the reaction was first order and cytochrome oxidase activity could be expressed as the velocity constant of the reaction. The optical density of oxidized cytochrome *c* was subtracted from the readings at the various time intervals and the logarithms of these differences were plotted against time. This gave a straight line from which a velocity constant $\frac{(\Delta \log \text{ ferrocytochrome } c)}{\Delta T}$ could be calculated (Cooperstein and Lazarow, 1950). Blanks (cytochrome *c* + homogenization medium) were carried out with each experiment in order that a correction could be made for the slow autoxidation of reduced cytochrome *c*.

In the present experiments the interest is in relative enzyme activities; accordingly all results are expressed as velocity constants. As can be seen from the cali-

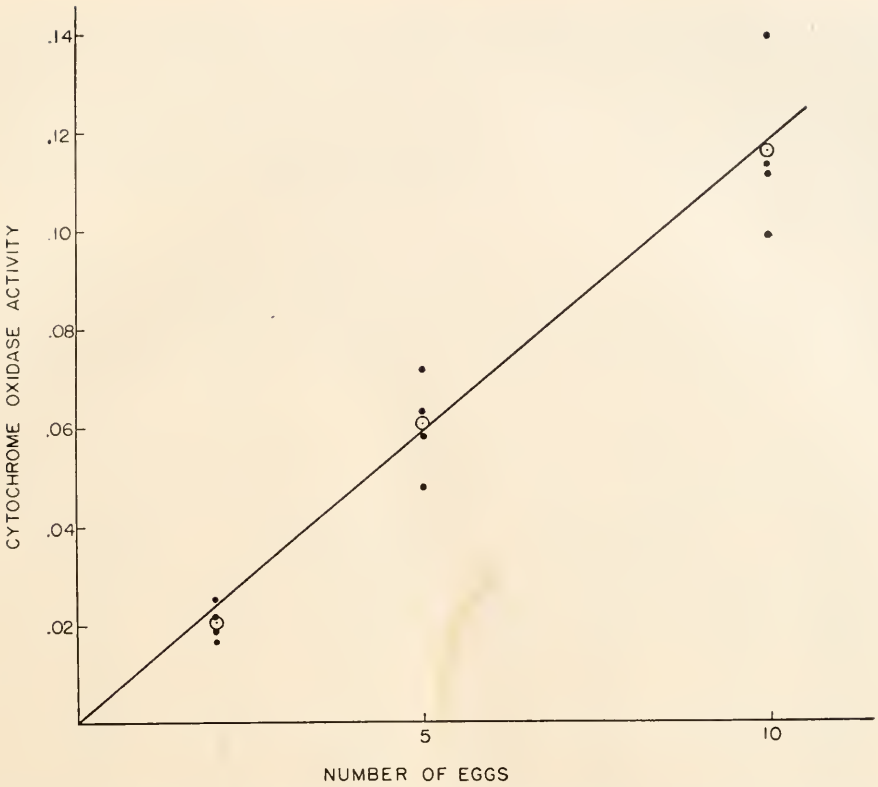

FIGURE 1. Cytochrome oxidase activity (Δ log ferrocytochrome *c*/min.) in homogenates of *Ciona* eggs. Solid points are individual measurements, large circles are averages.

bration curve (Fig. 1) these are proportional to the amount of cytochrome oxidase. The method is extremely sensitive, and it is possible to measure the cytochrome oxidase activity in a single *Ciona* egg.

RESULTS

Anterior and posterior blastomeres of *Ciona* were separated, and the cytochrome oxidase activities measured with the above described method. From previous work in determining the calibration curve it was decided that a sample containing about 20 second-cleavage blastomeres provided sufficient cytoplasm for reasonably accurate measurement of enzyme activity. In the first experiments 15 to 20 each of the anterior and posterior blastomeres were used, and the results were satisfactory. Straight lines representing reaction rates were obtained, and there was little subjective error in determining the slopes of the lines. As more skill in separating blastomeres was developed, the number used for each test was increased.

Several precautions were necessary in measuring the cytochrome oxidase of the blastomeres. A freshly prepared reduced cytochrome *c* solution tends to give high values for a standard cytochrome oxidase preparation (in these experiments, a

standard number of whole, unfertilized eggs). As the preparation ages, over a period of several hours, the values for the standard decrease. For comparative measurements of enzyme activity this error can be eliminated by running the samples simultaneously using several cuvettes, or it can be corrected by alternately measuring single samples. The average of the values should then eliminate the error due to a change of the stock cytochrome *c* solution. The latter method was chosen as it is more convenient and permits better standardization of the homogenization procedure.

In all experiments the freshly prepared cytochrome *c* was tested several times with whole eggs before it was used with the isolated blastomeres. The time between determinations of the enzyme in the two types of blastomeres was shortened by carrying the experimental procedure for both types of blastomeres up to the point of homogenization. The results, tabulated in Table I, are expressed as

TABLE I

Cytochrome oxidase activity in homogenates of anterior and posterior blastomeres of Ciona

Number of blastomeres	Cytochrome oxidase ($10^2 \Delta$ log ferrocytochrome *c*/min.) in anterior blastomeres	Cytochrome oxidase ($10^2 \Delta$ log ferrocytochrome *c*/min.) in posterior blastomeres	Ratio cytochrome oxidase activities $\left(\frac{\text{post.}}{\text{ant.}}\right)$
20	8.0	14.8	1.9
15	2.0	4.2	2.1
24	5.4	15.3	2.8
24	4.7	11.3	2.4
27	2.4	10.7	4.5
32	4.1	14.5	3.5
36	5.9	10.9	1.9
32	5.0	15.3	3.1
32	7.8	14.0	1.8
			average 2.7

velocity constants which are proportional to cytochrome oxidase activity (Fig. 1).

Since the majority of experiments were carried out under varied conditions (different batches of eggs, cytochrome *c* solutions, temperatures, etc.) it is not possible to compare velocity constants of separate experiments with any degree of reliability. The data in Table I must therefore be considered as a paired series with the results best expressed as ratios (shown in the last column of Table I). The cytochrome oxidase activity in posterior blastomeres averages 2.7 times that in anterior blastomeres, a difference which is sufficiently great to eliminate the need for a statistical test for significant difference. These results cannot be due to volume differences since the second cleavage is an equal one. The average diameters, as measured with an ocular micrometer, of 75 anterior and 91 posterior blastomeres were 103 microns and 104 microns, respectively.

DISCUSSION

As shown in Table I, the ratios in enzyme activity between the blastomeres vary from 1.8 to 4.5. To what extent this range of values reflects experimental error

is difficult to determine. The data in Figure 1 give some indication of accuracy although not all these measurements were made consecutively. A test for reliability of the method gave very good results; thus, in a series of determinations using five eggs each time, the velocity constants were 0.083, 0.074, 0.073, 0.083, 0.076. Ultramicro methods are particularly subject to erratic and inexplicable errors, and the present method is no exception. One very probable source of error is the homogenization procedure which, due to the very small amounts of material, is difficult to standardize. These and other errors, however, tend to be eliminated by averaging the results of separate experiments, and it is felt that the average value in Table I is reasonably accurate.

These results have considerable value in support of the cytochemical studies which have been carried out on the ascidian egg. Ries (1937, 1939), Reverberi and Pitotti (1939), Urbani and Mistruzzi (1947), using the indophenol reaction, demonstrated a localization of indophenol blue oxidase (presumably cytochrome oxidase) in posterior blastomeres of various ascidian embryos. The present results are free from the objections which may be raised against cytochemical techniques; however, as it turns out, they offer a striking confirmation of the earlier cytochemical results.

The indophenol reaction has also been used with other types of eggs and embryos, principally by Child (1941) and his students. Thus differential appearance of color with the reagents has been demonstrated in sea urchin, amphibian, and chick embryos. The results presented here indicate that the use of this cytochemical test is valid, and that localization or gradients of cytochrome oxidase probably exist in these other forms.

As is known from studies on other types of cells, the cytochrome oxidase complex is located in mitochondria and presumably this is true in the *Ciona* egg. The localization of cytochrome oxidase in the particulate fraction of centrifuged homogenates of *Ciona* eggs and the action of sodium desoxycholate on crude homogenates lend support to this assumption. The results, therefore, indicate a marked localization of mitochondria in the posterior blastomeres.

From cell lineage studies of the ascidian egg it is known that the myoplasm, the region rich in cytochrome oxidase, gives rise to the muscles of the tadpole. Conklin (1931) demonstrated that displacement of the pigmented inclusions from the myoplasm of *Styela* eggs did not alter subsequent differentiation of muscles. Similarly located "mitochondria" in *Ciona* eggs were not essential for muscle formation. Although Conklin refers to these inclusions as mitochondria, he reserves the possibility that they may be lipid granules due to their staining properties and their low density as indicated by displacement to the centripetal end of centrifuged eggs.

There are indications that these granules lack cytochrome oxidase which is an additional reason for not referring to them as mitochondria. In centrifuged eggs the indophenol reaction is absent in the centripetal region (Ries, 1939; Urbani and Mistruzzi, 1947). Experiments carried out in this laboratory demonstrate that the cytochrome oxidase-containing elements have a high density. Egg homogenates (50 eggs in 5 μl. phosphate buffer) were fractionated by centrifugation and the cytochrome oxidase measured by the microspectrophotometric method. The light fraction composed of oil droplets and small granules, presumably the "mitochondria" described by Conklin, contained little or no cytochrome oxidase whereas

the heavier fraction contained nearly all the enzyme. In another experiment a solid mass of eggs (about 5 μl.) with no excess fluid was homogenized and fractionated by centrifugation. Samples of the light fraction consisting of oil droplets and small granules showed little cytochrome oxidase activity as compared to a high oxidase activity of the heavier fractions.

It seems reasonable to conclude that the inclusions which Conklin described in the myoplasm of the ascidian egg are not cytochrome oxidase-containing mitochondria and therefore their lack of influence on muscle differentiation has no bearing on whether a high oxidase activity is necessary for such differentiation.

Experiments which are more pertinent to this question are those of Ries (1939) who concluded that the cytochemically demonstrated oxidases were essential for normal differentiation of muscles. The region of the centrifuged egg which gave rise to muscles always coincided with that which gave a strong oxidase reaction. Furthermore if the reactive cytoplasm was displaced by centrifugation, the muscles differentiated in an abnormal region. In addition Reverberi and Pitotti (1939) relate the presence of the oxidases to normal cleavage and development of bilateral symmetry.

These authors, quite rightly, do not consider their experiments as indicating that oxidases are the primary cause of differentiation. It seems obvious that if there is a segregation during cleavage of particulate elements such as mitochondria, we cannot assume that our indicator of this segregation, such as cytochrome oxidase, is in itself the cause of differentiation. Particulate elements are complex in structure and function, all of which factors would have to be considered with respect to differentiation rather than a single component.

Mitochondria are generally considered to be the "power plants" of the cell and supply energy for cellular activities. The potential for energy production of posterior blastomeres might be related to subsequent differentiation, although at an early stage their energy requirement, as measured by oxygen consumption, cannot be more than 20% different from that of anterior blastomeres (Holter and Zeuthen, 1944). It is evident that more information is needed before we can be specific in relating differentiation to a localization of specific enzymes, or even, as the results suggest, a differential distribution of mitochondria.

Summary

Anterior and posterior blastomeres were separated in the four-cell stage of *Ciona intestinalis* and cytochrome oxidase activities of homogenates of the blastomeres were quantitatively determined by a microspectrophotometric method. The average cytochrome oxidase activity of homogenates of posterior blastomeres is 2.7 times that of anterior blastomeres. The results indicate a localization of mitochondria in the posterior blastomeres.

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